DECREASED HEPATIC DRUG METABOLISING ENZYME ACTIVITY IN RATS WITH NITROSAMINE-INDUCED TUMOURS

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SUMMARY

N-Methyl *N*-benzyl nitrosamine (MBNA), which requires P450-dependant activation to be mutagenic, has been shown to produce squamous cell carcinoma of rat oesophagus. The aim of this study was to determine the effects of tumour induction on hepatic cytochrome P450 (CYP) and phase II enzyme activity. Female Wistar rats were given MBNA (2.5 mg/kg) by gavage, twice weekly for 12 weeks. At the end of 12 weeks they were sacrificed; livers and oesophagi were removed. The activity of hepatic CYP and phase II enzymes was determined by incubation of liver microsomes with appropriate CYP substrates. All rats receiving MBNA developed oesophageal lesions. Hepatic CYP1A2 activity (phenacetin 5 μM) in tumour-bearing rats was significantly decreased to 53% of the controls ($p < 0.05$). CYP2E1 (*p*-nitrophenol hydroxylase), CYP2D (debrisoquine hydroxylase) and CYP3A (quinine hydroxylase) activity was significantly ($p < 0.05$) reduced. Microsomal UDP-glucuronosyl transferase activity was also found to be markedly decreased while glutathione-*S*-transferase activity remained almost unchanged. Alteration of the activities of drug metabolising enzymes in rats with chemically induced tumours

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could be an important factor in determining resistance or susceptibility to xenobiotics and antitumour drugs.

KEY WORDS

nitrosamine, oesophageal tumour, cytochrome P450, drug metabolising enzymes

INTRODUCTION

Carcinogenesis in humans and animals is a complex, multistage process. Variation has been observed in the response of different animal species and strains to the same chemical carcinogen, in terms of both the site of tumour formation and the number of tumours produced. Such variation may suggest different mechanisms of action. Most of the available results refer to differences in the activation or detoxification of carcinogens.

Nitrosamines are among the most potent chemical carcinogens known and are believed to be carcinogenic to humans /1-3/. They are also one of the most intensively studied of the chemical carcinogens, and their peculiar biological effects (organotrophy, interspecies differences) have fascinated researchers for many years. Many nitrosamines demonstrate pronounced organ and species specificity /4/. For example, *N*-dimethyl nitrosamine is a potent hepatic carcinogen, *N*-methyl *N*-butyl nitrosamine is a bladder, oesophageal and hepatic carcinogen and *N*-methyl *N*-benzyl nitrosamine is a potent oesophageal carcinogen in rats /1,3/. Their organ specificity is caused in part by metabolic activation in target organs /5/. Methylbenzyl nitrosamine (MBNA) has been shown to transform oesophageal cells in culture, and rats treated with MBNA develop squamous cell carcinoma of the oesophagus, which is histologically very similar to human oesophageal carcinoma /6/. Squamous cell carcinoma of the oesophagus is one of the most common cancers world-wide /7,8/.

Whereas some inherently electrophilic chemicals may function as direct carcinogens, most DNA-damaging carcinogens act by forming reactive intermediates, generated by metabolic reactions in the body, which are capable of binding covalently to the DNA of cells from which tumours arise. Most of the enzyme systems involved in

carcinogen bioactivation are concentrated in the liver, but many extra-hepatic organs and tissues have appreciable quantities of such enzymes. A number of existing examples support the idea that metabolic difference is an important cause of species variation in carcinogenicity /9/.

Like many other xenobiotics, nitrosamines are believed to require metabolic activation in the host organism to exert their carcinogenic and/or toxic effects /10/. MBNA requires cytochrome P450 (CYP) dependant activation to be mutagenic. The first step of such metabolism is believed to be α-oxidation to form α-hydroxy-nitrosamines /11/. The newly-formed α-hydroxy-nitrosamines are unstable products with a half-life of seconds under physiological conditions. They decompose rapidly to form alkyl diacetates and aldehydes, both of which are believed to alkylate DNA /11/.

This study was conducted to determine the effect of MBNA administration on the hepatic drug and carcinogen-metabolising enzyme activities, which, in turn, could influence the effect of various cancer-chemopreventive agents as well as cancer-chemotherapeutic agents. There are a variety of factors affecting the response of tumours towards antitumour drugs including the content and activity of various xenobiotic-metabolising enzymes in liver and in tumour cells, expression of multi-drug resistance genes and target enzymes /11/. Expression of different xenobiotic-metabolising enzymes in liver and in tumour cells has been implicated as a factor possibly influencing both intrinsic and acquired drug resistance in tumours /11/.

MATERIALS AND METHODS

Chemicals and reagents

N-Methyl *N*-benzyl nitrosamine (NMBA) was synthesised by a nitrosation reaction to the corresponding secondary amine, *N*-benzyl methylamine, as previously described /12/. The purity of NMBA was approximately 95% determined by mass spectroscopic analysis. All other chemicals and reagents used were of HPLC analytical grade. Sodium dodecyl sulphate, hexan-1-ol, sodium hydroxide, potassium dihydrogen orthophosphate, potassium chloride, HPLC-grade acetonitrile, tert-methylbutyl ether, diethyl ether and methanol were purchased from BDH Chemicals (Poole, UK). Tetrabutylammonium

bromide, NADPH, sodium dithionite, *p*-nitrophenol, phenacetin, paracetamol, 2-aminophenol, glutathione and 1-chloro-2,4-dinitrobenzene were purchased from Sigma Chemical Co. (St Louis, MO, USA). Quinine hydrochloride was purchased from Merck-Schuchardt (Schuchardt, Germany). Disodium hydrogen orthosphosphate was purchased from Ajax Chemicals (NSW, Australia). 3-Hydroxyquinine was a gift from Dr. P Winstanley, University of Liverpool, UK. Debrisoquine and 4-hydroxydebrisoquine were kindly donated by Roche Ltd. (Auckland, New Zealand). Guanoxan hemisulphate was supplied by Pfizer Ltd. (Auckland, New Zealand).

Treatment of rats with *N*-methyl *N*-benzyl nitrosamine

The study was approved by the Otago University Animal Ethics Committee, Dunedin, New Zealand. Fifteen female Wistar rats, weighing 200-225 g and 8-9 weeks old (obtained from the Animal Breeding Station, Dunedin, New Zealand), were divided into two groups: control (n = 5) and treatment (n = 10). They were housed in hanging wire cages in a temperature-controlled room with a 12-hour light-dark cycle and were maintained on a commercial rodent diet (R-94, Reliance Stock Food Company, Dunedin, New Zealand), to which they had free access. The treatment group of rats were given *N*-methyl *N*-benzyl nitrosamine (MBNA) in olive oil by gavage at a dose of 2.5 mg/kg body weight twice a week for 12 weeks consecutively while the control group received only vehicle solvent (olive oil). Rats were weighed at the start of the experiment and subsequently at the end of each week to monitor whether treatment with MBNA affected their growth rate. At the end of 12 weeks treatment the animals were euthanized by CO_2 asphyxiation. Livers were removed immediately and washed in ice-cold normal saline, the excess saline blotted off and stored at -80°C until required. The oesophagi were removed, cut open longitudinally and fixed on corkboards. Tumour lesions were counted using a dissection microscope and then the oesophagi were rolled on fine wooden sticks, fixed in 10% buffered formalin for 24 h, and slides were prepared.

Liver microsomes were prepared by a differential centrifugation method /13/ and stored in phosphate buffer (pH 7.4) containing 20% w/v glycerol at -80°C until used. Microsomal protein content and P450 content were determined by the method of Lowry *et al.* /14/ and Omura and Sato /15/, respectively.

Determination of CYP1A2 activity

An HPLC method developed by Tassaneeyakul *et al.* /16/ was used to determine phenacetin-*O*-deethylase activity as a probe for CYP1A2. Activity of the high affinity component (CYP1A2) of phenacetin-*O*-deethylase was determined by incubating phenacetin (5 μM) at 37°C with liver microsomes (0.5 mg/ml) and measuring the formation of metabolite, paracetamol.

Determination of CYP3A activity

Quinine was used as a marker for determining CYP3A activity. The reaction mixture (0.5 ml) was incubated at 37°C and contained 30 μM quinine, NADPH, and liver microsomes (0.5 mg protein/ml). The HPLC procedure used for analysis of the formation of 3-hydroxy-quinine metabolite was that previously described by Wanwimolruk *et al.* /17/.

Determination of CYP2E1 activity

CYP2E1 activity was determined using an HPLC method, previously developed in the authors' laboratory, using *p*-nitrophenol as substrate. In brief, the reaction mixture containing *p*-nitrophenol (140 μM), rat liver microsomes (0.8 mg/ml), ascorbic acid (1 mM) and NADPH (1 mM) in a total volume of 0.5 ml phosphate buffer (pH 7.4) was incubated at 37°C for 25 min. After terminating the reaction using perchloric acid (0.6 M), an internal standard (phenacetin 1.5 μg/ml) was added. The metabolite formed, i.e. *p*-nitrocatechol, and the internal standard were extracted into tert-methylbutyl-ether after alkalinising with ammonium sulphate. The residue obtained, after evaporating off the ether, was analysed using an HPLC system consisting of a reversed phase column (C18 5 μm, 150 mm x 4.6 mm i.d.), mobile phase (acetonitrile:water: 16:84 v/v, pH 3.0) and a UV detector (243 nm).

Determination of CYP2D activity

Debrisoquine 4-hydroxylase (CYP2D) activity was measured using a method as previously described /18/. Briefly, each reaction mixture (1 ml) consisted of debrisoquine (substrate, 60 μM), rat liver micro-

somal protein (1 mg/ml) and NADPH (1.2 mM). Guanoxan hemisulphate (98 μM) was used as the internal standard. Analysis of the metabolite formed (4-hydroxydebrisoquine) was performed using an HPLC system consisting of a CN column (Spherisorb-CN, 5 μm, 15 cm x 4.6 mm i.d), mobile phase (acetonitrile:phosphate buffer: 12:88 v/v, pH 5) and UV detector (214 nm).

Determination of glucuronosyl transferase activity

Microsomal glucuronosyl transferase activity was determined using two different substrates, *p*-nitrophenol and 2-aminophenol. UDP-glucuronosyl transferase activity towards *p*-nitrophenol was determined using the spectrophotometric method described by Luquita *et al.* /19/ with slight modifications. *p*-Nitrophenol (800 μM) was used as a substrate for the assay. A membrane perturbant Triton-X 100 (0.05 mg/protein) and a β-glucuronidase inhibitor, D-saccharic acid lactone (0.5 mM), were systematically incorporated in the reaction medium. For determining conjugating activity towards 2-aminophenol, the method described by Burchell /20/ was used.

Determination of glutathione-*S*-transferase activity

Hepatic cytosolic glutathione-*S*-transferase activity was determined using a spectrophotometric method based on the enzyme-catalysed condensation of glutathione with the model substrate 1-chloro-2,4-dinitrobenzene /21/.

Statistical analysis

An SPSS package (version 8.1 for Windows 95) was used for all statistical analysis. Results are expressed as means ± standard deviation (SD). The differences between groups were evaluated using one-way analysis of variance (ANOVA). Bonferroni's procedure was used for multiple comparisons. In all cases, $p < 0.05$ was considered as the minimum level of statistical significance.

RESULTS

All rats treated with MBNA developed oesophageal tumours. One rat had to be euthanized 5 days before the end of the 12-week

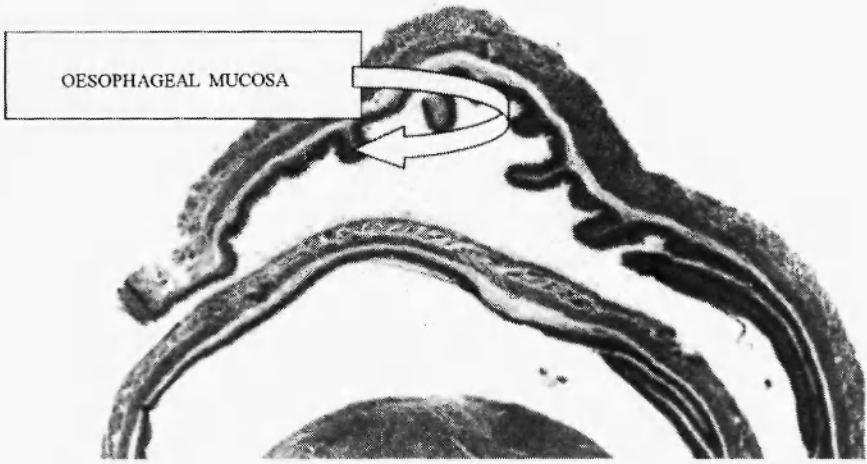
treatment period when tumours proximal to the pharynx made breathing difficult. In the treatment group, the tumour lesions were seen as irregular swellings, multiple exophytic tumours arising from the oesophageal epithelium, mostly ovoid in shape, with white verrucous surfaces, oriented along the axis of the oesophagus, while the control rats did not develop any tumours (Figs. 1, 2). No ulceration was noted in any part of the oesophagus in any of the rats. All organs, including the liver, did not show any histopathological changes. Treatment with MBNA did not significantly affect the body growth rate or liver weight. CYP content in the MBNA-treated rats was slightly less than that in the control group; however, this was statistically insignificant (Table 1). The activity of various CYP isoforms decreased quite considerably (Table 1). Activity of CYP1A2 in the MBNA-treated rats was significantly less (i.e. 53% of the control) than that observed in the control animals. CYP2E1 activity in

TABLE 1

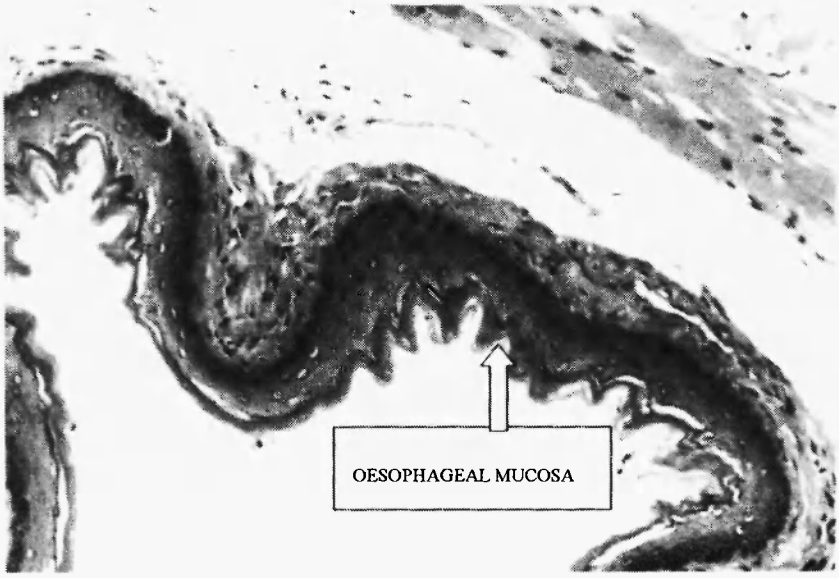
Content of hepatic CYP and activity of various hepatic drug metabolising enzymes in the rats with tumours and control rats. Rats in the tumour group were given MBNA (2.5 mg/kg) by gavage for 12 weeks.

	Control group	Tumour group
CYP content (nmol/mg)	0.21 ± 0.04	0.17 ± 0.06
CYP1A2 (nmol/mg/min)	0.084 ± 0.027	0.046 ± 0.02*
CYP2E1 (nmol/mg/min)	0.57 ± 0.06	0.27 ± 0.06*
CYP2D (nmol/mg/min)	0.91 ± 0.18	0.56 ± 0.09*
CYP3A (nmol/mg/min)	0.121 ± 0.03	0.074 ± 0.03*
UDPGT(PNP) (nmol/mg/min)	7.8 ± 3.1	3.3 ± 1.6*
UDPGT(2AP) (nmol/mg/min)	0.52 ± 0.06	0.34 ± 0.07*
Glutathione-*S*-transferase (μmol/mg/min)	0.23 ± 0.05	0.22 ± 0.02

Values are means ± SD.
* Significantly different from the control group ($p < 0.05$).
UDPGT(PNP) = UDP-glucuronosyl transferase activity towards *p*-nitrophenol;
UDPGT(2AP) = UDP-glucuronosyl transferase activity towards 2-aminophenol.

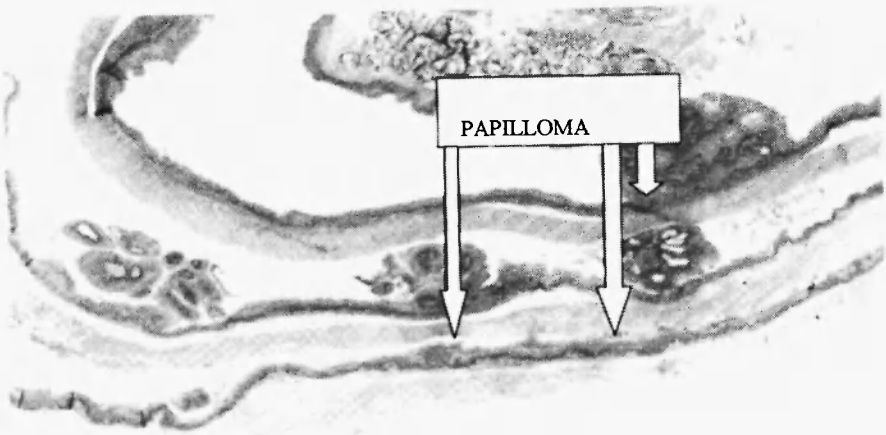


(a)



(b)

Fig. 1: Transverse section of oesophagus of control rats showing normal epithelial lining [magnification: (a) x 2, (b) x 4].



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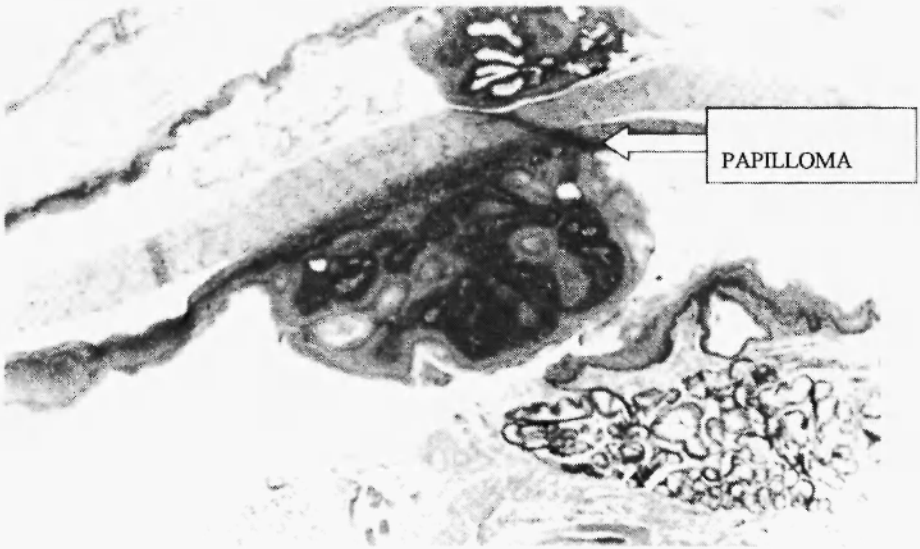

(b)

Fig. 2: (a) Overview of tumour lesion arising from the oesophageal epithelium of rats receiving MBNA for 12 weeks. (b) One of the papillomas in the close-up view is enlarged 10 times.

the MBNA-induced tumour rats was reduced to 48% of the control value ($p < 0.05$). Debrisoquine hydroxylase (CYP2D) and quinine hydroxylase (CYP3A) activities significantly ($p < 0.05$) declined to 63% and 61% of the control values, respectively.

For phase II conjugating enzymes (Table 1), UDP-glucuronosyl transferase activity towards *p*-nitrophenol conjugation was significantly lower ($P < 0.05$) in tumour-bearing rats than that in the control group. Activity of the enzyme in tumour-bearing rats was 42% of that of the controls. Similarly, UDP-glucuronosyl transferase activity towards 2-aminophenol conjugation was significantly different between the two groups. However, activity of glutathione-*S*-transferase in the tumour-bearing rats was similar to that seen in the control group.

DISCUSSION

The present study has demonstrated organ specificity of MBNA-induced tumours in rats. All rats had developed oesophageal tumours after 12 weeks of treatment with MBNA. MBNA-treatment did not cause any hepatic preneoplastic foci or liver tumours. Furthermore, other parts of the gastrointestinal tract, namely, stomach, intestine and colon, were devoid of any tumours or other pathological changes. This observation is consistent with previous reports /22,23/. As nitrosamines are considered to be carcinogenic in humans, exposure to environmental and/or endogenously-formed nitrosamines and dietary exposure to *N*-nitroso-compounds may be a risk factor /24/. Experimental studies have revealed generation of nitrosamines from nitrites and secondary amines in acid media, both *in vitro* and in the rat stomach /25/.

In this study, it is interesting to note that, in most of the CYP isoforms studied, as well as UDP-glucuronosyl transferase, activity was significantly decreased in rats consequent to 12 weeks treatment with the carcinogenic nitrosamine, MBNA. There is good evidence to indicate that several different isoforms of CYP may play an important role in bioactivation of different alkylating agents, such as *N*-nitroso-compounds /26/. Therefore, any alteration in the activity of CYP isozymes could affect various deleterious biological consequences of these agents in individuals at high risk of exposure to any carcinogenic agents that might exist in the environment. Metabolites of chemical

carcinogens are capable of binding to DNA, correlating well with their carcinogenic potencies, and depending mainly on their enzymatic conversion processes mediated by CYP enzymes /26/. It has been reported previously that short-term pre-treatment of rats with carcinogenic nitrosamines, such as dimethylnitrosamine or *N*-nitrosomorpholine, increased phenobarbitone sleeping time, and decreased the content of hepatic microsomal protein level and CYP /26/. *In vitro* studies have shown that CYP-mediated metabolism of aniline, ethylmorphine and *p*-nitrobenzoic acid was decreased in rats with nitrosamine-induced tumours /27/.

Habib *et al.* /28/ studied the effect of treatment with one tenth of the LD_{50} as a single dose of various volatile *N*-nitroso-compounds on the metabolism of carcinogen-metabolising enzymes in mouse liver. All of the *N*-nitrosamines investigated, namely *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosodipropylamine, *N*-nitroso-methylamine, *N*-nitrosomethylbutylamine and *N*-nitrosoethylbutylamine, markedly increased the hepatic content of CYP and cytochrome b_5, and the activities of NADPH-cytochrome *c* reductase and aryl hydrocarbon hydroxylase.

The reasons for differences in the observation of changes in CYP content as well as other metabolising enzymes between the present study and the above-mentioned study could be explained by taking into account (a) dose and frequency of the carcinogen exposure, and (b) presence or absence of tumour induction in rats. In the study of Habib *et al.* /28/, only a single dose of nitrosamine was administered and livers were collected after 4 h. This relates to a scenario of an "acute one-off dose exposure" to the carcinogen, while, in the present study, multiple doses were given for a sufficiently long period of 12 weeks to induce tumours. This would correspond more to a "repeated or chronic situation". Repeated exposure to a chemical could have a significantly different effect on induction or inhibition of xenobiotic-metabolising enzymes in comparison to a single exposure. It is yet to be determined whether short-term positive effects are consistently maintained in the long-term. The initial induction effect might be transient, level off and decrease during continuous and constant insult by the same chemical on the same enzymes. At least one study has reported that suppression of CYP isozymes was reversed upon long-term exposure to a metabolism-modifying agent, phenylhexyl isothiocyanate /29/. It is also possible that long-term exposure to

MBNA at the dose used in the present study might have caused toxicological changes in the rat liver, negatively affecting its biochemical functioning, yet not causing any obvious pathology in hepatic cells. It has been reported that diethylnitrosamine, which causes hepatocarcinogenesis in rats, reduced microsomal CYP enzymes to 50% and the CYP1A2-mediated activation of heterocyclic amines to 20% of the controls /30/.

The significant decrease in hepatic phase I and II enzyme activities, observed here, could have important implications for tumour induction by MBNA on the metabolism and, hence, subsequent disposition of various chemotherapeutic as well as chemopreventive agents. However, this claim requires ratification by experiments substantiating the decrease in specific CYP isoforms, such as immunohistochemical or molecular biological methods. It may be reasonable to state that activation and detoxification of a drug or carcinogen will depend on its presence, the amount present, and the extent of activity of xenobiotic-metabolising enzymes in liver and other tissues. The human CYP3A subfamily plays a role in the metabolism of these drugs, including cyclophosphamide, ifosfamide, etoposide, tamoxifen, taxol and vinca alkaloids /31/. Involvement of CYP2D6 in metabolism of doxorubicin and vinblastine has also been reported /31/. It should be recognised that many anticancer agents have active metabolites which, depending on their potency and pharmacokinetics, may contribute to clinical response. Cyclophosphamide and ifosfamide are alkylating anticancer agents that in humans require biotransformation by CYP3A to produce active cytotoxic compounds. Such is also the case with vinblastine, which is converted to active desacetylvinblastine by CYP3A /31/. Moreover, phase II conjugating enzymes are also important in the detoxification of antineoplastic agents. Any enhancement or inhibition of activity of these enzymes could result in increased or decreased capacity for their detoxification by conjugation reactions. There have been reports of CYP3A4 and CYP2E1 involvement in the bioactivation of carcinogenic nitrosamines such as *N*-nitrosonornicotine and *N*-nitrosodimethylamine /32/. This might also suggest a potential role of these enzymes in the metabolic activation of other nitrosamines as well.

Alteration of the content and activity of xenobiotic-metabolising enzymes in rats with chemically induced tumours may be an important factor in determining resistance or susceptibility to xenobiotics or

antitumour drugs. Both the amount and proportion of different enzymes present in the liver and tumour tissues play a role in determining anti-cancer drug resistance. Further studies to determine expression of these enzymes in liver as well as in tumour cells would be beneficial in understanding drug resistance in depth. Individual CYPs could be regulated in a complex manner, given that cancer susceptibility of an individual might relate to alteration in CYP-related catalytic activities. In humans, variable but considerable inter-individual differences are observed in the profiles of carcinogen-metabolising enzymes, which may be due to genetic polymorphism, or the result of diet, or exposure to drugs or environmental chemicals. Though direct extrapolation of the present results into the human situation is difficult without further evidence, they point to the possibility of variation in the metabolism and, hence, the therapeutic response of antineoplastic agents in patients with tumours, compared to healthy individuals.

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